ASSOCIATION OF rs2073498 ALA133SER GENE POLYMORPHISM WITH RISK TOWARDS LUNG CANCER SUSCEPTIBILITY IN A NORTH INDIAN POPULATION

A

Thesis submitted

In partial fulfillment for the award of the

Degree of Master of Science in Biotechnology

BY

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CERTIFICATE

This is to certify that the thesis entitled **"Association of rs2073498 Ala133Ser gene polymorphism with risk towards lung cancer susceptibility in a North Indian population"** being submitted by **Ms. Harinder Kaur** (Roll No. 301101014) in partial fulfillment of the requirement for the award of Master of Sciences in Biotechnology, Thapar University, Patiala, is a bonafide work carried out under the esteemed supervision of **Dr. Siddharth Sharma,** Assistant Professor, Department of Biotechnology & Environmental Sciences (DBTES), Thapar University.

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DECLARATION

I hereby declare that the work being presented in the thesis entitled **"Association of rs2073498 Ala133Ser gene polymorphism with risk towards lung cancer susceptibility in a North Indian population"** in partial fulfillment of the requirements for the award of degree of Masters in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to June 2013, under the conception and supervision of **Dr. Siddharth Sharma**, Assistant Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. This dissertation has not been submitted elsewhere for the award of any degree or diploma by other universities.

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LIST OF ABBREVIATIONS

APC - Anaphase promoting complex

ATM - Ataxia telangiectasia mutated

BAD - Bcl-2-associated death promoter

BAX - Bcl-2–associated X protein

BCR – B-cell receptor

BH3 - Bcl-2-homlogy domain 3 (BH3)

BSA - Bovine serum albumin

C19ORF5/MAP1S - Chromosome 19 open reading frame 5/microtubule-associated protein 1S

CNK1 – Connector enhancer of KSR like proteinDNA - Deoxyribonucleic acid

EDTA - Ethylene diamine tetra cyclic acetic acid

EGF – Epidermal growth factor

GTP/GDP – Guanosine triphosphate/Guanosine diphosphate

IARC - International agency for research on cancer

KSR – Kinase suppressor of Ras

MAPK - Mitogen activated protein kinase

MOAP-1 - Modulator of apoptosis

Nore - Novel ras effector

PI3-K - Phosphatidylinositol 3-kinase

PKC - Protein kinase c

RAS - Rat Sarcoma

SCLC - Small cell lung carcinoma

TAZ - Transcriptional coactivator with PDZ-binding motif

TBE - Tris-Chloride Boric acid EDTA

TE - Tris-Chloride EDTA

TNF-R1 - TNFα receptor 1

TTF-1 – Thyroid transcription factor-1

YAP – Yes associated protein

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ABSTRACT

The tumor suppressor gene *RASSF1A* (Ras association domain family 1 isoform A) regulates cell cycle regulation, apoptosis and microtubule stability and suffers from frequent allelic loss and gene silencing via promoter hypermethylation in a variety of human malignancies. An association between a single nucleotide polymorphism (SNP) at codon 133 of the *RASSF1A* gene, encoding either alanine (GCT) or serine (TCT), and human lung cancer risk in a North-Indian population was studied by investigating the distribution of the Ala133Ser SNP in 90 patients with lung cancer and 95 healthy controls by polymerase chain reaction and restriction enzyme-digestion assay. The individuals with Ser/Ser genotype showed increased risk of lung cancer, though data was statistically insignificant due to small size of population studied (OR: 2.03, 95% CI; 0.35-11.55, *p* value = 0.41). Risk of SQCC development was found to be significantly high in carriers of the Ser/Ser mutant alleles of Ala133Ser polymorphism when compared with homozygote Ala/Ala genotype (OR: 2.9, 95% CI; 1.25-6.80, *p* value=0.01).

INTRODUCTION

Cancer is the general name for a group of more than 100 diseases in which abnormal cells divide without control**.** It is a potentially fatal disease caused by both external as well as internal factors that mutate genes encoding critical cell-regulatory proteins. The resultant aberrant cell behaviour leads to expansive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated disease. Cancer was the leading cause of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008 (Globocon 2008, WHO). According to IARC, lung cancer is the most frequent cancer type, followed by breast, colorectum, stomach and prostate cancer respectively.

TABLE 1: Globocon Data Fact Sheets, 2008

World	**Male**	**Female**	**Both sexes**
Population (thousands)	3414566	3358715	6773281
Number of new cancer cases (thousands)	6617.8	6044.7	12662.6
Risk of getting cancer before age 75 (%)	21.1	16.5	18.6
Number of cancer deaths (thousands)	4219.6	3345.2	7564.8
Proportion (per 100,000)	550.6	620.8	585.8

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The treatment of cancer requires a careful selection of one or more of the major treatment modalities – surgery, radiotherapy and systemic therapy – a selection that should be based on evidence of the best existing treatment given the resources available. Surgery alone, and sometimes radiation alone, is only likely to be highly successful when the tumor is localized and small in size. Chemotherapy alone can be effective for a small number of cancers, such as hematological neoplasms (leukemias and lymphomas) (WHO, 2013). There is increasing emphasis worldwide on the development of specialized cancer centers that apply evidence-based multimodal therapies and provide rehabilitation and palliative care.

With more than 1.1 million deaths annually worldwide, lung cancer is the most frequent and one of the most deadly cancer types. Lung cancer can be divided into two broad groups: small cell lung cancer (SCLC) accounts for about 20-25% of bronchogenic carcinomas and non-small cell lung cancer (NSCLC) constitutes almost all of the remainder. SCLCs express properties of neuro-endocrine cells, whereas most NSCLCs lack these properties and are comprised of three major subtypes - adenocarcinoma, squamous cell carcinoma and large cell carcinoma.

There is an overwhelming evidence that tobacco smoking is the major cause of lung cancer in most human populations. The smoke inhaled by smokers of cigarettes and other tobacco products contains numerous carcinogens, as well as agents that cause inflammation. Other factors include exposure to respiratory carcinogens, genetic factors and history of other lung diseases.

Ras proteins play a direct casual role in human cancer with activating mutations in Ras occurring in almost 30% of tumors (Van Der and Adams, 2007). Ras effectors also contribute in cancer as mutations occur in Ras effectors. In 2000, a new Ras effector was identified, RAS-association domain family. The *RASSF* gene family comprises 10 members, termed *RASSF1* to *RASSF10*. *RASSF1* gene is located on the short arm of chromosome 3 (3p21.3). There are eight different *RASSF1* isoforms (*RASSF1A* to *RASSF1H*) that are generated by differential usage of two promoters and through alternative splicing. The *RASSF1* gene, a putative tumor suppressor gene, shows frequent allelic loss and gene silencing via promoter hypermethylation in a variety of human malignancies. *RASSF1A*/*RASSFIA* stands for Ras Association domain Family 1 Isoform A.

The tumor suppressor gene, *RASSF1A*, has been reported to play a role in diverse activities including cell cycle regulation, apoptosis and regulating microtubule dynamics as well as maintenance of genomic instability. Several tumor suppressor pathways are now known to be directly modulated by *RASSFIA*. *RASSF1A* suffers epigenetic inactivation in over 50% of human tumors (Damman *et al*., 2005; Donninger *et al.*, 2007).

In tumors and tumor cell lines, loss of expression is largely attributed to promoter hypermethylation. Though somatic mutations of *RASSFIA* are uncommon, several single nucleotide polymorphisms (SNPs) have been identified in the gene (Gordon and Baksh, 2011; Van der Wayden and Adams, 2007). One of these polymorphisms, a guanine(G)/thymine(T)

common single nucleotide polymorphism at first position of codon 133 in exon 3 of *RASSFIA* (dbsnp ID: rs2073498) resulting in the substitution of an alanine (Ala) residue (GCT) by serine (Ser) residue (TCT) in the ATM phosphorylation site has been demonstrated to affect *RASSFIA* function (El kalla *et al*., 2010; Shivakumar *et al.*, 2002), which subsequently refers towards its probable association with cancer risk.

REVIEW OF LITERATURE

2.1 Cancer

As described earlier, Cancer is a term used for group of diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph system (NCI, 2009). Cancer continues to be a worldwide killer, despite the enormous amount of research and rapid developments seen during the past decade. According to recent statistics, cancer was accounted for 7.6 million deaths in 2008, which represents 13% of total deaths (Globocon 2008, WHO). Causes of carcinogenesis are governed by both external and internal factors. External factors which can be categorised as carcinogens are tobacco, chemicals, radiations, fumes and smokes as well as exposure to some infectious organisms (ex- hepatitis C virus). Internal factors contributing to cancer development are inherited mutations, hormonal disturbances, immunity imbalances etc.

It is evident that most common cancers are largely preventable. Tobacco use still remains the most important risk factor and poses the greatest challenge. Cancer of the lung, mouth and pharynx and, to an extent, oesophagus is all associated with tobacco use. The cancers of these sites are preventable with reduction of morbidity and mortality. Primary prevention with implementation of tobacco control strategies is therefore possible. Adequate and balanced dietary practices, and changes in preservation and storage practices of food have prevented cancers of the stomach and oesophagus.

2.2 Lung Cancer : An Introduction & Classification

Lung cancer arises when a series of genetic mutations occur in a normal lung cells causing them to behave abnormally and reproduce endlessly (http://www.cancerresearchuk.org). The buildup of extra cells often forms a mass of tissue called a growth or tumor, which can spread by breaking away & travel through blood vessels or lymph vessels to reach other parts of the body. After spreading, cancer cells may attach to other tissues and grow to form new tumors that may damage those tissues (http://www.cancer.gov).

It is becoming apparent through candidate gene and genome wide approaches that clinically evident lung cancers have accumulated numerous (perhaps 20 or more) clonal genetic

and epigenetic alterations as a multistep process. These alterations include the classical genetic abnormalities of tumor suppressor gene (TSG) inactivation and overactivity of growth promoting oncogenes (Fong *et al*., 2003).

Clinically lung cancer can be categorized into two groups that reflect their biology and management:

a) Small cell lung cancer (SCLC) and
b) Non-small cell lung carcinomas (SCLC)

2.2.1 Small Cell Lung Cancers

About 10% to 15% of all lung cancers are small cell lung cancer (SCLC), named for the size of the cancer cells when seen under a microscope. Other names for SCLC are oat cell cancer, oat cell carcinoma, and small cell undifferentiated carcinoma (American Cancer Society, 2013). It's a malignant epithelial tumor consisting of small cells with scant cytoplasm, ill defined cell borders, finely granular nuclear chromatin, and absent or inconspicuous nucleoli. The cells are round, oval and spindle-shaped. Nuclear molding is prominent. Necrosis is typically extensive and the mitotic count is high. Symptoms include central location and locoregional spread. While small cell carcinoma is a light microscopic diagnosis, electron microscopy shows neuroendocrine granules approximately 100 nm in diameter in at least two-thirds of cases and immunohistochemistry is positive for CD56, chromogranin and synaptophysin in most cases (Nicholsan *et al.,* 2002). Less than 10% of SCLC are negative for all neuroendocrime markers. Small cell carcinoma is also positive for TTF-1 in up to 90% of cases (Guinee *et al., 1994)*. Thus we can say that SCLC and pulmonary carcinoids are classic neuroendocrine (NE) tumors and they reflect all of the characteristic features of NE cells.

2.2.2 Non-Small Cell Lung Cancers

Non-small cell lung cancer (NSCLC) accounts for 80% to 85% of all lung cancer cases and includes 3 major types: Adenocarcinoma, Squamous cell carcinoma and Large-cell carcinoma.

a) **Adenocarcinoma** : According to IARC , an adenocarcinoma is defined as "A malignant epithelial tumor with glandular differentiation or mucin production, showing acinar, papillary, bronchioloalveolar or solid with mucin growth patterns or a mixture of these patterns." Compared to other lung cancers, adenocarcinomas are most frequently peripheral nodules under 4.0 cm in size (Colby *et al*., 1995). They infrequently present in a central location as a hilar or perihilar mass and only rarely show cavitation. Pleura and chest wall involvement is seen in approximately 15% of cases and this is more frequent than with other forms of lung cancer. The malignant nuclei are ovoid with delicate smooth membranes and finely reticulated, evenly dispersed chromatin with small nucleoli and a single large nuclear pseudoinclusion. Cytoplasm is delicate and contains secretory vacuoles some of which contain mucin (IARC, 2008). According to WHO, adenocarcinoma can be further classified into following subtypes- Acinar. Papillary, Bronchioloalveolar carcinoma, Adenocarcinoma with mixed subtypes and other variants.

b) **Squamous Cell Carcinoma**: Squamous cell carcinoma (SQCC) is a malignant epithelial tumor showing keratinization and/or intercellular bridges that arises from bronchial epithelium (IARC, 2008). The majority of squamous cell lung carcinomas arise centrally in the mainstem, lobar or segmental bronchi (Tomashefski *et al*., 1990). The cytological manifestations of squamous cell carcinoma depend on the degree of histological differentiation and the type of sampling. In a background of necrosis and cellular debris, large tumor cells display central, irregular hyperchromatic nuclei exhibiting one or more small nucleoli with an abundant cytoplasm. Squamous cell lung carcinoma is characterized by high-level expression of keratin genes and histological evidence of keratinization. They are also characterized by over expression of p53 related gene p63 (Fuji *et al.,* 2002). Studies have demonstrated that the amount of EGF receptor is elevated in squamous cell carcinoma cells in tissue culture when compared with normal epidermal cells. Therefore, the level of EGF receptor may be an excellent marker for squamous malignancies (Hendler and Ozanne, 1984).

c) **Large Cell Carcinoma**: According to the definition by IARC, large cell carcinoma is an undifferentiated non-small cell carcinoma that lacks the cytological and architectural features of small cell carcinoma and glandular or squamous differentiation. Large cell carcinoma accounts for approximately 9% of all lung cancers in most studies. Large cell neureoendocrine carcinoma accounts for about 3% of lung cancer. All types predominate in smokers (Travis *et al.*, 1995). Most cases of LCC do not have specific discriminating cytological features. Most cytological samples show cellular aggregates; less often cells are dispersed. Cellular borders are indistinct so syncytia is formed haphazardly. Nuclei vary from round to extremely irregular with irregular chromatin distribution. Nucleoli are generally very prominent. Cytoplasm is basophilic, usually scant with a high nuclear-to-cytoplasmic (N/C) ratio (Burn *et al.*, 1969). Large cell carcinomas typically present as large, peripheral masses, frequently identified on chest radiographs, but which may also involve subsegmental or large bronchi. The tumor often invades visceral pleura, chest wall, or adjacent structures. Large cell carcinomas share the molecular and genetic alterations commonly seen in NSCLC, since it is a poorly differentiated tumor issued from the same stem cells, exposed to the same carcinogens. K-ras mutations, P53 mutations and Rb pathway alteration occurs with the same frequency as in other NSCLC. Large cell neuroendocrine carcinomas have P53 and Rb mutational patterns in addition to inactivation pathways similar to SCLC (Brambilla *et al.*, 1993; Brambilla *et al.*, 1999).

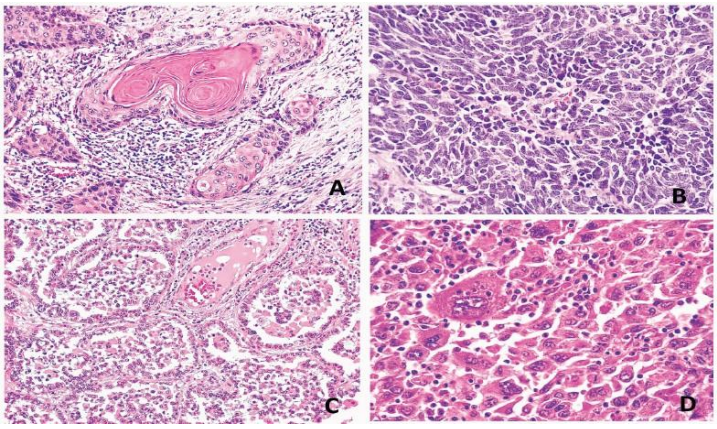


Fig.1 Different lung carcinomas. A. Squamous cell carcinoma; B. Small cell lung carcinoma; C. Adenocarcinoma; D. Large cell carcinoma (Source- Carcinoma of lung: Histopathology. *HCDCP* Feb 2nd, 2012).

2.3 Statistics : Lung Cancer in India

TABLE 2: Cancer Incidence and mortality data for all ages in Indian population. 5- year prevalence for adult population only.

Cancer	Incidence			Mortality			5-year prevalence		
	Number	(%)	ASR (W)	Number	(%)	ASR (W)	Number	(%)	Prop.
Lip, oral cavity	69820	7.4	7.5	47653	7.5	5.2	107690	6.3	13.4
Nasopharynx	3333	0.4	0.3	2412	0.4	0.2	8410	0.5	1
Other pharynx	45271	4.8	5	39346	6.2	4.3	66391	3.9	8.2
Oesophagus	48099	5.1	5.3	43351	6.8	4.8	25610	1.5	3.2
Stomach	35059	3.7	3.8	33564	5.3	3.6	25257	1.5	3.1
Colorectum	36476	3.8	3.9	25690	4.1	2.8	49122	2.9	6.1
Liver	20144	2.1	2.2	18043	2.8	2.0	9639	0.6	1.2
Gallbladder	17262	1.8	1.9	10279	1.6	1.1	21038	1.2	2.6
Pancreas	8960	0.9	1	7766	1.2	0.9	5052	0.3	0.6
Larynx	23058	2.4	2.5	14794	2.3	1.7	45753	2.7	5.7
Lung	**58567**	**6.2**	**6.6**	**52269**	**8.3**	**5.9**	**26994**	**1.6**	**3.3**
Melanoma of skin	945	0.1	0.1	483	0.1	0.1	2394	0.1	0.3
Breast	115251	12.1	22.9	53592	8.5	11.1	315679	18.5	80.6
Cervix uteri	134420	14.2	27	72825	11.5	15.2	338010	19.8	86.3
Corpus uteri	8772	0.9	1.9	4851	0.8	1.1	32013	1.9	8.2
Ovary	28080	3	5.7	19558	3.1	4.1	57796	3.4	14.8
Prostate	14630	1.5	3.7	10422	1.6	2.5	48892	2.9	11.8
Testis	3864	0.4	0.6	1665	0.3	0.3	12980	0.8	3.1
Kidney	8900	0.9	0.9	5733	0.9	0.6	18356	1.1	2.3
Bladder	14812	1.6	1.7	8203	1.3	1	33590	2	4.2
Brain, nervous system	21835	2.3	2.1	17941	2.8	1.8	31781	1.9	3.9
Thyroid	12899	1.4	1.2	3029	0.5	0.3	51521	3	6.4
Hodgkin lymphoma	7371	0.8	0.7	3587	0.6	0.4	11899	0.7	1.5
Non-Hodgkin lymphoma	23718	2.5	2.4	16243	2.6	1.7	27031	1.6	3.3
Multiple myeloma	6789	0.7	0.8	5941	0.9	0.7	11602	0.7	1.4
Leukaemia	33307	3.5	3	26282	4.1	2.5	24658	1.4	3.1

ASR (W) and proportions per 100,000.

ASR – Age standardized rate

Reference: GLOBOCON 2008 Cancer Fact Sheets, IARC. Estimated incidence, mortality and 5-year prevalence.

2.4 Role of SNP's in cancer

Cancer is a complex disease, like diabetes, heart disease, and kidney disease. All complex diseases arise from combinations of changes that occur in the same cell over a period of time. In cancer, these changes accumulate over many years. Only when a critical number of changes occur in the same cell does it finally become cancerous. To complicate matters further, there are many different types of cancer, each displaying different combinations of changes. This poses many questions in front of researchers, such as, how can researchers hope to identify and study all the changes that occur in so many different cancers? How can they explain why some people respond to treatment and not others? Scientists believe that tiny variations in the human genome called Single Nucleotide Polymorphisms, or SNPs (snips) for short, can help them to answer these questions. They believe SNPs can help them catalogue the unique sets of changes involved in different cancers. They see SNPs as a potential tool to improve cancer diagnosis and treatment planning. They suspect that SNPs may play a role in the different responses to treatments seen among cancer patients. And they think that SNPs may also be involved in the different levels of individual cancer risk observed.

An amazing aspect of the human genome is that of the 3.2 billion bases, roughly 99.9 percent are the same between any two people. It is the variation in only a tiny fraction of the genome, roughly several million bases, that makes a person unique. Variation occurs whenever the order of the bases in a DNA sequence changes. The outcome of variations depends on two factors: where in the genome the change occurs, (i.e., in a noncoding region, coding, or regulatory region) and the exact nature of the change. There are a group of variations in coding and regulatory regions that result in harmful effects. These are called mutations. They cause disease because changes in the genome's instructions alter the functions of important proteins that are needed for health. For example, diabetes, cancer, heart disease, Huntington's disease, and hemophilia all result from variations that cause harmful effects. In a "simple" disease such as hemophilia, variation in one gene is sufficient to cause disease symptoms. By contrast, in a "complex" disease like cancer, symptoms are seen only after many variations have occurred in different genes in the same cell.

The most common type of genetic variation is called a Single Nucleotide Polymorphism (SNP). A SNP is defined as a single base change in a DNA sequence that occurs in a significant proportion (more than 1 percent) of a large population. The single base is replaced by any of the other three bases. Here is an example: in the DNA sequence CAT, a SNP occurs when the A base changes to a C, and the sequence becomes CCT.

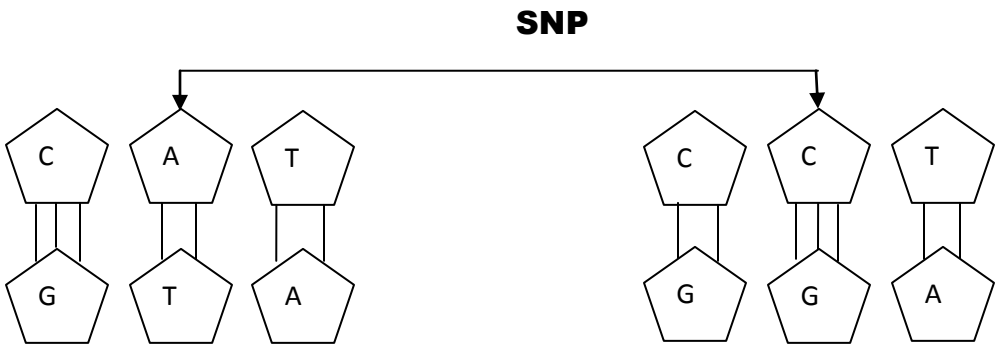


Fig. 2: **A Diagrammatic representation of Single Nucleotide Polymorphism**

SNPs are scattered throughout the genome and are found in both coding and noncoding regions. SNPs can cause silent, harmless, harmful, or latent effects. They occur with a very high frequency, with estimates ranging from about 1 in 1000 bases to 1 in 100 to 300 bases. This means that there could be millions of SNPs in each human genome. The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.

Most SNPs occur in noncoding regions and do not alter genes. If a SNP is frequently found close to a particular gene, it acts as a marker for that gene. The remaining SNPs occur in coding regions. When a SNP occurs within a coding region, it is possible that there is no effect. This type of change in the DNA is called a silent change. But if it alters the protein made by that coding region, this in turn could influence a person's health.

Because of SNPs, a person's genome may express a very active carcinogen-making protein, or a sluggish one, or something in between. A protein with a very active binding site can "grab" more precarcinogen than usual. Or the protein may convert the pre-carcinogen to the carcinogen at a faster rate. In both cases, more carcinogen molecules pile up in the lungs, causing

damage to the cells' DNA, which can lead to cancer. On the other hand, if the SNPs result in a slow carcinogen-making protein, the lung is exposed to fewer DNA-damaging carcinogens, and the chance of cancer is reduced. But until the body is exposed to cigarette smoke, no effect is seen. SNPs may also influence detoxifying enzymes that prepare carcinogens for elimination from the human body. SNPs that result in very active forms of detoxifying enzymes will remove the carcinogens quickly from the body, allowing less time for damage. SNPs that produce sluggish detoxifying enzymes will permit carcinogens to remain in the body for a longer time, thus aiding in occurrence of cancer.

Since SNPs can be gene markers, SNP profiles may help scientists to identify the full collection of genes that contribute to the development of complex diseases such as cancer. Armed with data from the SNP Map, cancer researchers across the country are looking for correlations between SNPs and precancerous conditions, SNPs and drug resistance in chemotherapy, SNPs and cancer susceptibility, and SNPs and drug response. Scientists are also using SNPs to calculate risk factors associated with cancer in large populations (http://www.cancer.gov, National Cancer Institute at National Institutes of Health).

2.5 ***RASSFIA*: Introduction, Key interactions & its role in human tumorigenesis**

Ras proteins play a direct causal role in human cancer with activating mutations in Ras occurring in ~30% of tumors. The Ras GTPases are a superfamily of molecular switches that regulate a diverse range of functions, including cell proliferation, differentiation, motility and apoptosis in response to extracellular signals. The Ras proteins exist in two states: a GTP-bound active state and a GDP-bound inactive state. In its GTP-bound state, Ras is able to interact with its downstream effectors, and mediate some component of Ras' cellular actions through complex signal transduction cascades. Ras effectors are proteins that specifically bind the GTP-bound form of Ras via the Ras protein effector domain. Ras effectors also contribute to cancer, as mutations occur in Ras effectors, notably B-Raf and PI3-K, and drugs blocking elements of these pathways are in clinical development (Van Der and Adams, 2007).

2.5.1 Ras signalling pathway

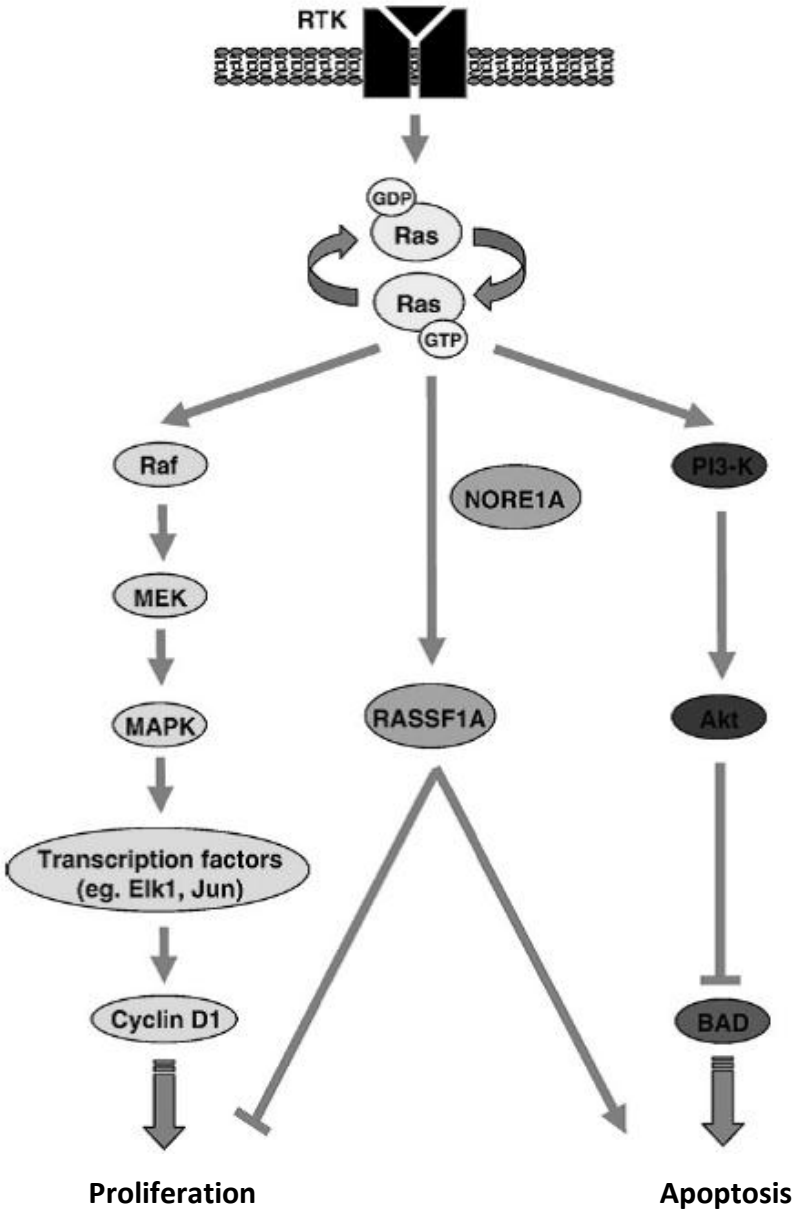


Fig. 3: **Ras signaling pathways**

Figure 3 shows the signaling pathways of Ras proteins. Ras transmits signals from receptor tyrosine kinases (RTK) to the nucleus and regulate a diverse array of biological functions. Ras functions as a molecular switch, being inactive when bound to GDP and active when bound to GTP. Activated Ras acts by regulating the cellular response through distinct Ras effectors

proteins and their complex signal transduction cascades, such as mediated by the Raf serine/threonine kinases, the lipid kinase, phosphatidylinositol 3-kinase (PI3-K) and the Ras association domain family 1, *RASSF1A*. The best-characterised signal transduction pathway of Ras is by the Raf kinases. Activated Raf phosphorylates MAPK/ ERK kinase (MEK) and the activated MEK phosphorylates the mitogen activated protein kinase (MAPK), which becomes activated and translocates to the nucleus where it phosphorylates a set of transcription factors. For example, the activation of Elk-1 leads to the transcription of Fos, which together with the MAPK-activated Jun, forms the activation protein 1 (AP-1), which has been shown to induce cyclin D1 and therefore stimulate proliferation. Another cascade of Ras-activated signalling is by anti-apoptotic PI3-K, which can stimulate the activity of the protein kinase B, Akt. Akt subsequently phosphorylates BAD, a pro-apoptotic member of the Bcl-family, and thus inhibits apoptosis (inactivating BAD enables BCL to promote cell survival by blocking the release of mitochondrial cytochrome c and therefore inhibiting caspase activation). Additionally, Ras regulates a pro-apoptotic pathway by binding to the Ras effectors *NORE1* and *RASSF1A* and *RASSF1A* can also block cell cycle progression.

2.5.2 Ras Association Domain Family

The Ras-Association Domain Family comprises ten members from *RASSF1* to *RASSF10* as well as various isoforms, which are listed in Fig. 4. One characteristic features of this family is the Ras-association domain (RA), which can be found either C-terminally (*RASSF1* to *RASSF6*) or N-terminally (*RASSF7* to *RASSF10*). The other characteristic feature is the Sav–RASSF–Hpo (SARAH) domain, encoding a protein–protein interaction domain, which however is only found in *RASSF1* to *RASSF6*. Prominent and most intensely studied family members are *RASSF1A* (an isoform of *RASSF1*) and *RASSF5*, also called *NORE1*. Whereas *RASSF7* to *RASSF10* joined the family only recently and therefore little data exist to date (Richter *et al.,* 2009). They seem to modulate some of the growth inhibitory responses mediated by Ras and may serve as tumor suppressor genes. Thus, RASSF proteins are tumor suppressors, in contrast to traditional Ras effectors such as Raf and PI3-K, which are oncoproteins (Van Der and Adams, 2007).

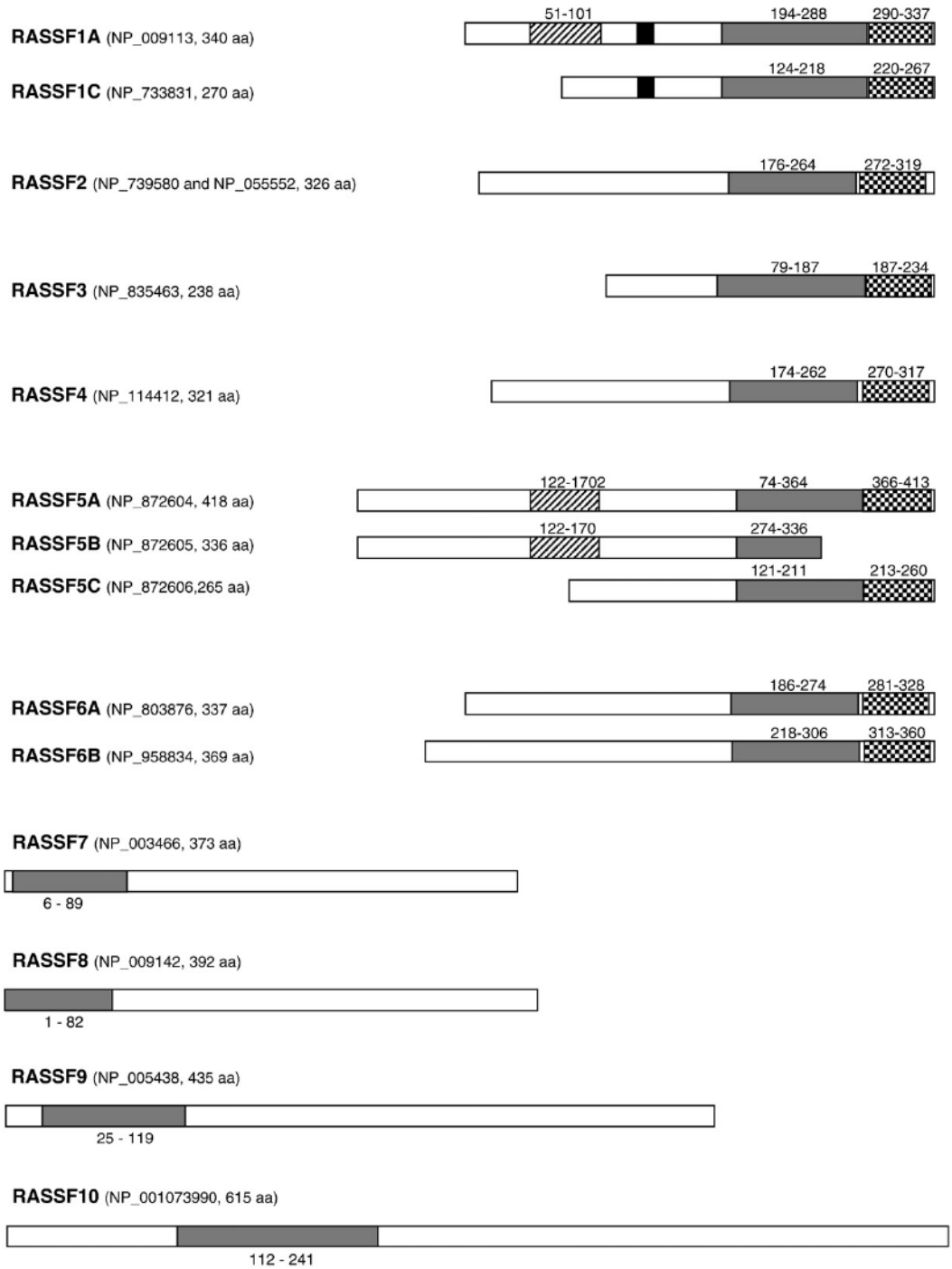


Fig. 4: **The Ras-Association Domain Family (*RASSF*).** Family members are listed according to Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez) with accession number and length in amino acids (aa). Conserved protein domains were determined with Prosite (http://www.expasy.ch/prosite/). Characteristic domains are the protein kinase C conserved region (C1; striped), the putative ATM-kinase phosphorylation site (black), Ras-association (RalGDS/AF-6) domain (RA; grey) and the Sav–*RASSF*–Hpo interaction site (SARAH; checkered) (Richter *et al.*, 2009).

2.5.3 ***RASSFI* : Introduction & Isoforms**

The *RASSF1* gene, which is located on the small arm of chromosome 3 (locus 3p21.3) codes for eight exons (1α, 1β, 2αβ, 2γ, 3, 4, 5 and 6) spanning approximately 11 kb (Dammann *et al.,* 2000) (Fig. 5). There are seven different *RASSF1* isoforms (*RASSF1A* to *RASSF1G)* that are generated by differential usage of two promoters (distance 3.5 kb) and through alternative splicing (Richter *et al.,* 2009). *RASSF1A* and *RASSF1C*, ubiquitously expressed in normal tissues, are the major isoforms. The isoform A is being transcribed from the upstream promoter and isoform C from the downstream promoter. These isoforms have four common C-terminal exons (exons 3–6) which encode a RalGDS/AF6 or Ras-association (RA) domain (Yamamoto *et al.,* 1999) and a Sav/*RASSF*/Hpo (SARAH) domain. RA domains mediate interactions with Ras and other small GTPases, and SARAH domains mediate protein–protein interactions crucial in the pathways that induce cell cycle arrest and apoptosis. Exon 3 also contains a putative ataxia telagectasia mutant (ATM) kinase phosphorylation consensus sequence motif (a peptide containing this sequence is phosphorylated in vitro, suggesting that *RASSF1*, like p53, may be a substrate for ATM (Kim *et al.,* 1999). The putative DAG binding (C1) domain can only be found in *RASSF1A*. (Fig. 6)

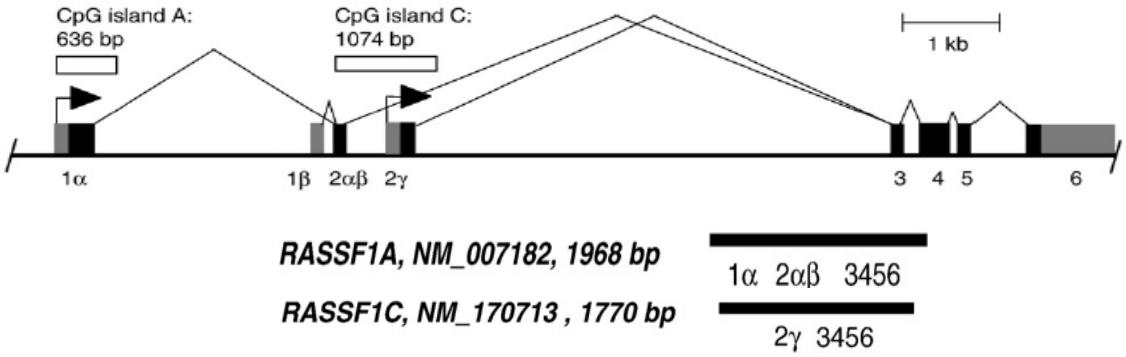


Fig. 5: **Genomic arrangement of *RASSF1* at 3p21.3.** Organization and transcripts are depicted according to Entrez Gene (www.ncbi.nlm.nih.gov/ sites/entrez) with accession numbers and length in bp. Black and grey boxes represent coding and untranslated sequences, respectively. Transcription start sites are marked by arrows. Location and size of CpG islands at promoter regions were determined by CpG plot (www.ebi.ac.uk/emboss/cpgplot/). *RASSF1* codes for eight exons termed 1α , 1β , 2αβ , 2γ, 3, 4, 5 and 6. The transcripts are spliced into seven isoforms *RASSF1A-RASSF1G. RASSF1A* and *RASSF1C* are transcribed from two distinct CpG island promoters. (Richter *et al.,* 2009)

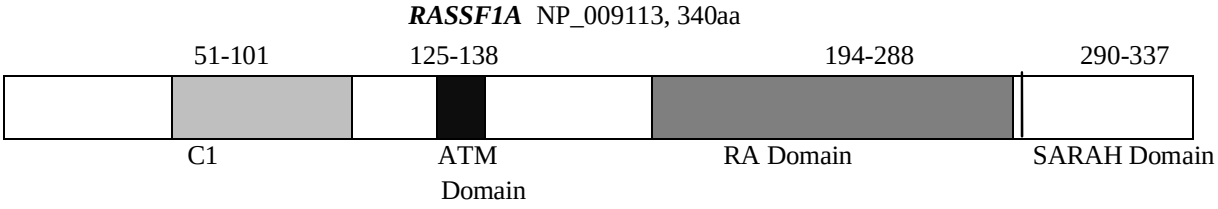


Fig. 6: **The *RASSFIA* gene**. Conserved protein domains were determined with Prosite (http://www.expasy.ch/prosite/). Characteristic domains are the protein kinase C conserved region (C1), the putative ATM-kinase phosphorylation site, Ras-association (RalGDS/AF-6) domain (RA) and the Sav–RASSF–Hpo interaction site (SARAH).

2.5.4 *RASSFIA* : Key interactions

RASSF1A is a tumor suppressor that suffers epigenetic inactivation in over 50% of human tumors (Dammann et al., 2005; Donninger et al., 2007; Van Der and Adams, 2007). Tumor suppressor genes are classically defined by Knudson's 'two-hit' hypothesis (Knudson, 1971), which states that inactivation of both alleles of a tumor suppressor gene is required for tumorigenesis. Loss of a *RASSF1A* allele is a frequent phenomenon in primary human cancer (Burbee et al., 2001; Pfeifer and Dammann, 2005). *RASSF1A* is pro-apoptotic and can induce cell cycle arrest in G2 and in G1 (Shivakumar et al., 2002; Vos et al., 2004, 2006). *RASSF1A* has no enzymatic activity, but appears to act by modulating microtubule polymerization and by scaffolding other tumor suppressors. Several tumor suppressor pathways are now known to be directly modulated by *RASSF1A* including MOAP-1/Bax (Baksh et al., 2005; Vos et al., 2006), the hippo pathway (Guo et al., 2007), and p53 (Song et al., 2008). The Key *RASSFIA* interactions are listed below.

a) *RASSF1A* & K-Ras

RASSF1A contains a Ras association domain and a cysteine rich domain (CRD). By analogy to the classic Ras effector Raf, both of these domains have the potential to bind directly to Ras onco- proteins (Drugan *et al*., 1996). The homology of *RASSF1A* with the mammalian RAS effector *NORE1* suggested that the *RASSF1A* gene product may function in signal transduction pathways involving RAS-like proteins. Recent data indicate that *RASSF1A* itself binds to RAS only weakly, and that binding to RAS may require heterodimerization of *RASSF1A*

and *NORE1* (Ortiz-Vega *et al*., 2002). *RASSF1A* has been linked to RAS signaling and it has been proved that interaction of PMCA4b (plasma membrane Ca^{2+} pump4b) with *RASSF1A* significantly inhibited the EGF-dependent activation of the mitogen-activated protein kinase ERK (Armesilla *et al.*, 2004). *RASSF1A* can be detected in an endogenous complex with Ras (Calvis *et al*., 2006) where it can form a complex with exogenously expressed activated K-Ras and serve as pro-apoptotic K-Ras effector (Vos *et al*., 2006; Donninger *et al*., 2007). There remains some controversy over the physiological nature of the interaction between Ras and *RASSF1A*. This may have arisen because only the K-Ras specific isoform interacts with *RASSF1A*, and the K-Ras protein must be post-translationally modified to support the interaction (Donninger *et al*., 2007).

b) *RASSFIA* & Microtubules

Earlier work showed that *RASSF1A* forms a complex with the microtubule network in cells and co-immunoprecipitates with most tubulin isoforms (Donninger *et al.,* 2011). The microtubule stabilizing effect of *RASSF1A* in interphase cells and the effects of its overexpression on mitotic cells are surprisingly similar to those produced by the commonly used cancer chemotherapy drug paclitaxel (taxol), which produces mitotic arrest by stabilization of microtubules leading to a loss of microtubule dynamics (Jordan *et al*., 1993). The domain required for both microtubule association and stabilization was mapped to a 169 amino-acid fragment that contains the RAS association domain. Yeast two-hybrid studies have found that *RASSF1A* can directly bind a series of microtubule associating proteins (MAPS) including MAP1a/MAP1b, C19ORF5 (Dallol *et al*., 2004), and MAP4 (Vos *et al*., 2004). Thus, it seems likely that the association with microtubules is indirect. Interestingly, certain tumor derived mutants of *RASSF1A* appear to lose the ability to associate with specific tubulin isoforms (El-Kalla *et al*., 2010). Moreover, deletion mutants of *RASSF1A* that lose the ability to complex with micro- tubules are defective for the induction of cell cycle arrest (Vos *et al*., 2004). These data suggest that the interaction of *RASSF1A* with microtubules may be unusually complex and essential for its tumor suppressing activity.

c) *RASSF1A* & MOAP-1/Bax

MOAP-1 is a BH3 (Bcl-2 homology3) domain containing protein that binds and activates the pro-apoptotic effector Bax (Tan et al., 2005). It was reported that *RASSF1A* is required for death receptor-induced BAX conformational change and apoptosis (Baksh *et al*., 2005). After receptor stimulation *RASSF1A* and the modulator of apoptosis-1 (MOAP-1 or MAP-1; a BH3-like protein) are recruited to the membrane and form a complex. When *RASSF1A* binds MOAP-1, this releases MOAP-1's intramolecular inhibition, and MOAP-1 is then able to associate with BAX (Baksh *et al*., 2005). It was further shown by that the binding of *RASSF1A* to MOAP-1 is enhanced in the presence of activated K-Ras. *RASSF1A* activates BAX through MOAP-1 and activated KRAS, *RASSF1A* and MOAP-1 synergize to induce BAX activation and cell death (Vos *et al.,* 2006). *RASSF1A* was found to be involved in the death receptor-dependent apoptosis pathway (extrinsic pathway). Upon tumor necrosis factor alpha (TNFα) stimulation, MOAP-1 associates with the TNF receptor 1, *RASSF1A* is recruited to this complex and *RASSF1A* homodimerization is lost prior to the recruitment. Therefore, *RASSF1A* is linked to death receptor-dependent apoptosis.

d) *RASSF1A* and the Hippo pathway

RASSF1A directly binds and activates the pro-apoptotic MST1 and MST2 kinases (Praskova *et al*., 2004; Avruch *et al*., 2006). These kinases drive the hippo pathway by phosphorylating and kinases LATs1 and LATs2 (Harvey and Tapon, 2007). These kinases then phosphorylate the transcription factors YAP and TAZ, which in turn activate the p73 tumor suppressor (Matallanas *et al*., 2007). The full role of *RASSF1A* in this pathway may be more complex as it also directly binds a protein called Salvador. Salvador may serve as an adaptor to promote the phosphorylation of LATs by MST (Guo *et al*., 2007). However, Salvador also appears to be a tumor suppressor in its own right, with significant functions independent of the classic hippo pathway (Donninger *et al*., 2011).

In summary, we now know that *RASSF1A* can complex with multiple known tumor suppressors and modulate their activity. This gives *RASSF1A* the potential to serve as a tumor suppressor node, integrating the activity of multiple tumor suppressors and connecting K-Ras to their action.

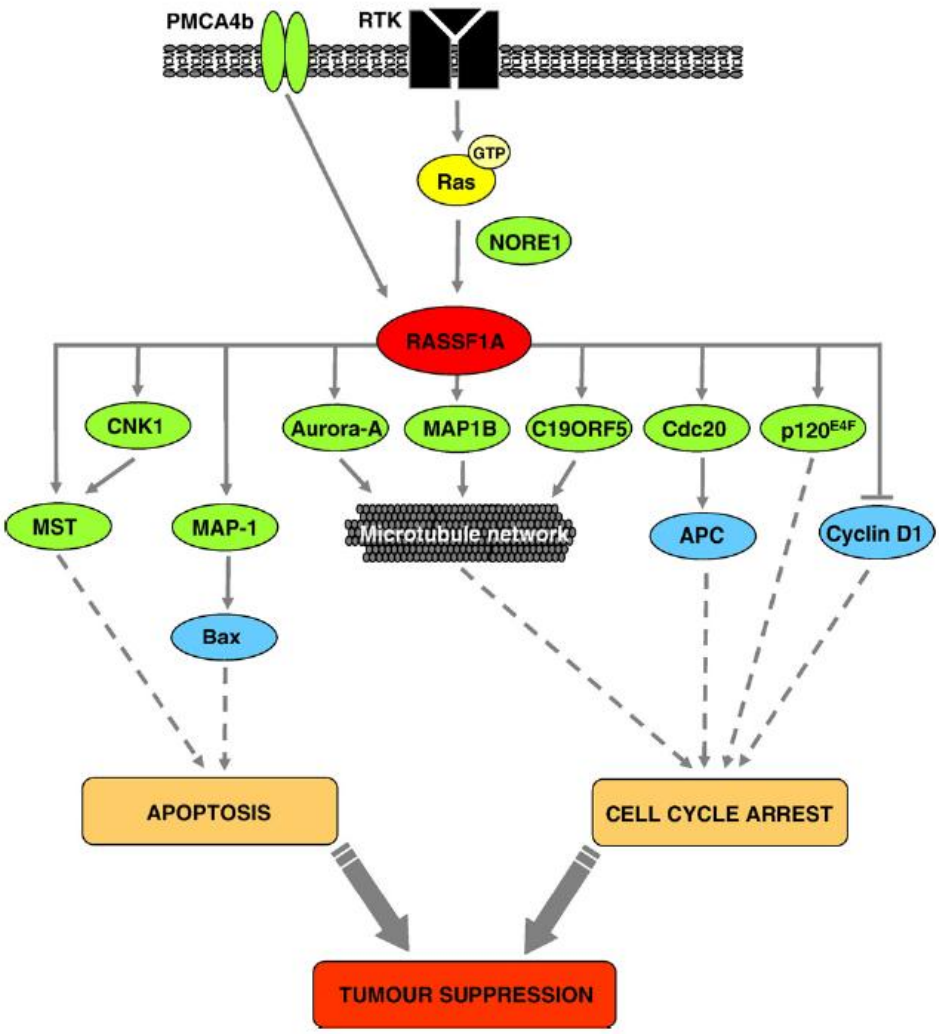


Fig. 7: A summary of the reported *RASSF1A* interactions and *RASSF1A*-mediated biological functions. *RASSF1A* can regulate the microtubule network, cell cycle progression and apoptosis by recruiting effectors and their signalling pathways. Proteins that directly interact (bind) with *RASSF1A* are shown in green, with downstream proteins affected by this interaction shown in blue. *RASSF1A* induces apoptosis through its interaction with Ras, the Ras effector *NORE1*, the connector enhancer of KSR (CNK1), the pro-apoptotic kinase MST1, and the modulator of apoptosis-1 (MAP-1; activated K-Ras, *RASSF1A*, and MAP-1 synergize to induce Bax activation and cell death). *RASSF1A* regulates proliferation through its interactions with the microtubules and Cdc20 (by inhibiting the APC–Cdc20 complex and its degradation of cyclins A and B), the microtubule-associated protein 1B (MAP1B), Aurora-A (which phosphorylates *RASSF1A*), C19ORF5 (the C19ORF5– *RASSF1A* interaction at the centrosome is thought to be required for the proper control of the APC–Cdc20 complex during mitosis),

the transcription factor p120E4F (*RASSF1A*-induced G1 cell cycle arrest and S-phase inhibition was enhanced by p120E4F) and inhibition of cyclin D1 accumulation. *RASSF1A* also inhibits the epidermal growth factor-dependent activation of Erk through the plasma membrane calmodulin-dependent calcium ATPase 4b (PMCA4b). Taken together, these activities all support a tumor suppressor role for *RASSF1A*. (Van Der and Adams, 2007)

2.6 Polymorphisms in *RASSFIA*

RASSF1A is one of the most epigenetically silenced elements in human cancers. Although silencing is done by promoter-specific methylation in cancers, several somatic nucleotide changes (polymorphisms) have been identified in *RASSF1A* in tissues from cancer patients. It is speculated that both nucleotide changes and epigenetic silencing result in loss of the *RASSF1A* tumor suppressor function and the appearance of enhanced growth. *RASSF1A* polymorphisms have been identified in several cancers. These polymorphisms have been found in tumors from numerous cancer patients and cell lines. The population distribution and significance of these alterations in tumorigenesis remain to be determined but do vary from 9% to 33% of the specific cancer population. The majority of *RASSF1A* polymorphisms have been confirmed using several approaches as outlined by the 1000 Genome project (http://www.1000genomes.org/), HapMap project (http://hapmap.ncbi.nlm.nih.gov/) and submitted by multiple sources. Kashuba *et al* (2009) determined that the mutation rate in cancer for *RASSF1A* was 0.42 mutation frequency/100 base pair whereas in the "normal" population was about 0.10 mutations/100 base pairs. They speculate that *RASSF1A* has a 73% GC content within exons 1–2 which may explain the high mutation rate of *RASSF1A* within cancer cells. Within cell lines, *RASSF1A* was found to carry 0.7 mutations/100 bp in the Burkitt's lymphoma-derived cell lines, BL2 and RAMOS, whereas it was 0.14 in the renal carcinoma cell line KRC/Y and, with each division of the BL2 lymphoma line, transitional mutations were observed. Interestingly, codon changes in *RASSF1A* were also observed in 15 normal human hearts that included two nucleotide changes (CTA to CTG and GTA to GTG) but no amino acid changes. They speculate that *RASSF* is simply located in an area that is "extensively damaged" and susceptible to mutational pressures in 90% of epithelial cancers (Gordon *et al*., 2012).

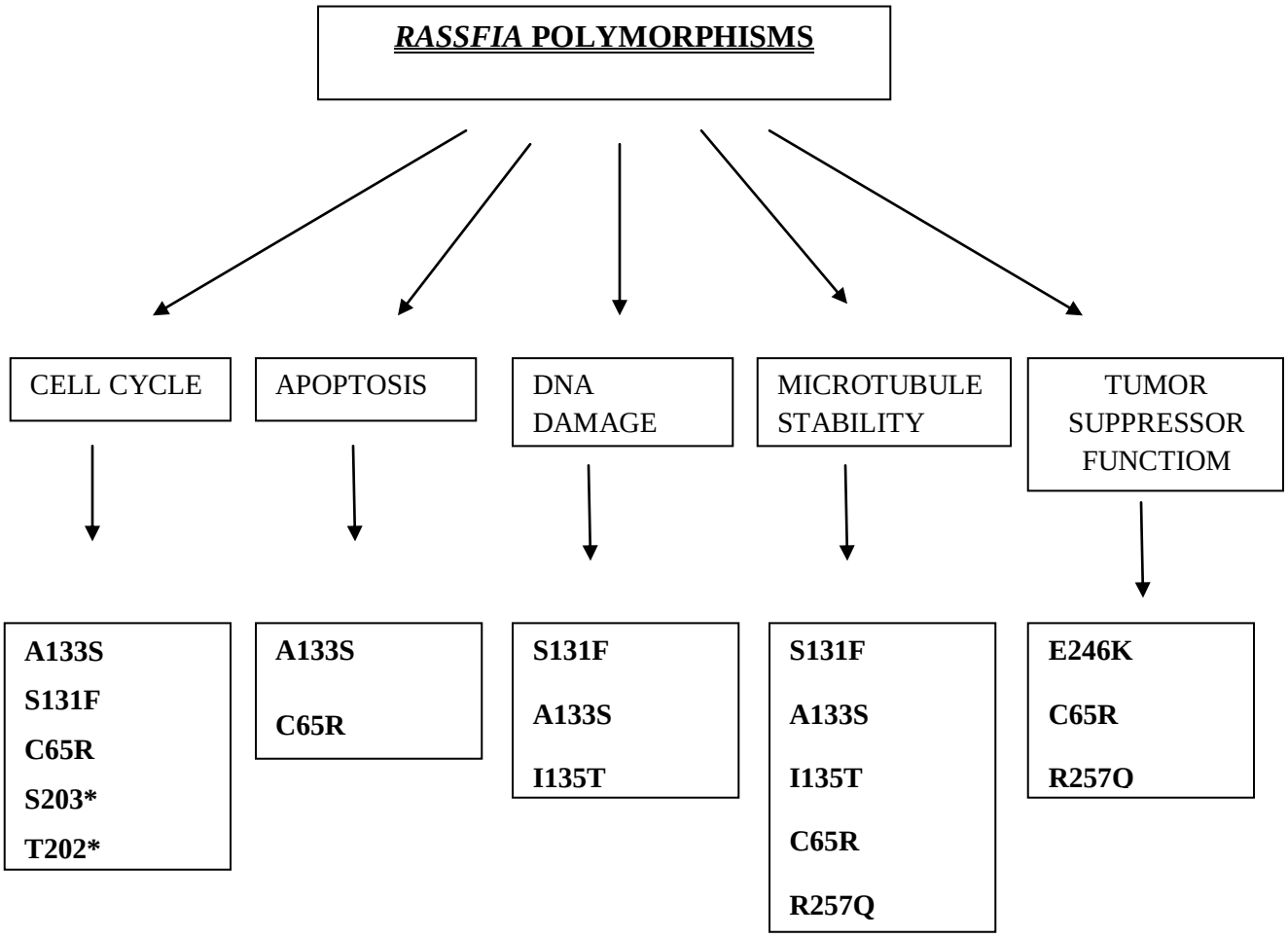


Fig. 8: Identified biological roles for *RASSF1A* polymorphisms. Several polymorphisms have been identified for *RASSF1A* over the past decade since it was first cloned. Biological analyses of the *in vivo* role have identified the importance of *RASSF1A* over numerous pathways. This figure summarizes what is known about *RASSF1A* polymorphisms. ∗ denotes a non-polymorphic but mutational change. This change does not naturally exist in the cancer patient population to our knowledge. (Gordon *et al.,* 2012).

2.7 The rs2073498A SNP

Several SNPs have been identified in the *RASSF1A* gene. The best characterized (rs2073498) is a G-T variation resulting in the presence of a Serine instead of an Alanine at amino acid 133 in the *RASSF1A* protein. This A(133)S variant protein has been associated with a reduced ability to regulate the cell cycle at G1/S (Shivakumar *et al.,* 2002). This Ala133Ser SNP is located at the bona fide phosphorylation site and very close to the amino-terminal domain (Kanzaki *et al.,* 2006). Although the A(133)S variant retains an overtly similar association with microtubules as the wild type protein, it is defective for the interaction with gamma and alpha (but not beta) tubulin (El-Kalla *et al*., 2010). Moreover, the change of Alanine133 to a Serine destroys a consensus phosphorylation site for the ATM kinase, an essential component of the DNA damage response (Hamilton *et al*., 2009). In fact it generates a new consensus phosphorylation site for several other kinases, including Casein kinase II. A significant increase in the degree of adhesion was detected in the A(133)S variant compared to the wild type *RASSF1A* expressing cells *in-vitro* (Kanzaki *et al.,* 2006). Thus, the biology and the regulation of the A(133)S variant may be significantly different to that of the wild type protein.

Several case–control studies have investigated the association between *RASSF1A* Ala133Ser polymorphism and risk of various cancers. An association between the *RASSF1A* Ala133Ser polymorphism and risk for various cancers inluding breast cancer (Gao *et al.,* 2008; Schagdarsurengin *et al.,* 2005) and lung cancer (Kanzaki *et al.,* 2006) has been reported. On the contrary, *RASSF1A* Ala133Ser polymorphism is not confirmed as a significant germline contributer to familial breast cancer susceptibility by Bergqvist *et al.* (2010). Moreover, Kanzaki *et al.* (2006) have found no significant association of *RASSF1A* Ala133Ser polymorphism and risk of colorectal cancer, head and neck cancer and esophageal cancer in Japanese population. Studies done on Turkish population revealed an association between risk of hepatocellular carcinoma and Ala133Ser polymorphism (Bayram , 2012).

OBJECTIVES

The objectives of this study are as following :-

1. To investigate the genotypic frequencies of the Ala133Ser SNP in lung cancer patients and controls in a North Indian population by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) assay.

2. To analyze the association between the Ala133Ser SNP towards lung cancer risk and also to evaluate the association of the above SNP with various clinico-pathological features using statistical analysis.

MATERIALS AND METHODS

4.1 Subjects

A case control study of 90 histo-pathologically confirmed lung cancer patients were recruited from the Post Graduate Institute of Medical Research, Chandigarh (PGIMER) and 95 cancer free controls from North Indian population were obtained. The study was ethically approved by the Institute ethics committee of PGIMER, Chandigarh. Written informed consent was obtained from all participants or from patients' representatives if direct consent could not be obtained. Each patient donated 5 ml venous blood upon admission to the hospital and was interviewed to collect demographic data and clinical information.

4.2 DNA Isolation from peripheral blood

Each of the samples were then processed as per the following protocol to isolate DNA from them. 5ml of blood samplewas taken in a falcon tube and 5ml of washing buffer (Table 3) was added into it. The mixture was thoroughly homogenised and then centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded and 5ml of washing buffer was added to the pellet .The pellet was resuspended in the buffer and centrifuged again This step was repeated thrice. Furtherthe pellet was dissolved in 5ml of Lysis buffer (Table 4) and incubated at 44 °C overnight.

Next day, an equal volume of Phenol: chloroform: Isoamyl alcohol mix (in a ratio of 25:24:1) was added to the tube contents and then both things were mixed slowly and thoroughly. The resultant solution was centrifuged at 8000 rpm for 10 minutes at 4°C. The upper aqueous layer was shifted to a new falcon tube and was again treated with the PCI mix and centrifuged. The resultant aqueous layer was shifted to another falcon tube and equal volume of Chloroform: Isoamyl alcohol mix (24:1) was added to it. The solution was centrifuged at 6500 rpm for 5 minutes and the resultant aqueous layer was again separated, to which equal volume of chilled isopropanol or 2.5 times volume of absolute ethanol was added and mixed thoroughly. The falcon was frozen at -20°C for 1-2 hours and later centrifuged at 12,000 rpm for 10 min at 4°C.The supernatant was discarded and the pellet of DNA was washed twice with chilled 70% ethanol by centrifugation at 10,000 rpm for 5 minutes. Ethanol was decanted and the pellet was

air dried. Finally, the dried pellet was dissolved in 50 to 100µl Tris-EDTA buffer, according to the size of pellet (Bartlett and White, 2003).

TABLE 3: Composition of Washing buffer

STOCK CONCENTRATION	**WORKING CONCENTRATION**	**VOLUME REQUIRED TO MAKE 5ml WASHING BUFFER***
1M sucrose	320 mM sucrose	1.6ml
100% Triton X-100	1% Triton X-100	0.05ml
100mM Magnesium Chloride	5Mm Magnesium Chloride	0.25ml
100mM Tris-HCl pH=8	10mM Tris-HCl pH=8	0.5ml

*****Volume was made upto 5ml by adding 2.6ml autoclaved water. .

TABLE 4: Composition of Lysis buffer.

STOCK CONCENTRATION	**WORKING CONCENTRATION**	**VOLUME REQUIRED TO MAKE 5ml LYSIS BUFFER[#]**
1M TrisHCl pH=8	400mM TrisHCl pH=8	2ml
10% SDS	1% SDS	0.5ml
0.5M EDTA	60mM EDTA	0.6ml
5M NaCl	150mM NaCl	0.15ml
10mg/ml Proteinase-K	100 µg/ml Proteinase-K	0.05ml

[#] Volume was made up to 5ml by adding 1.7ml autoclaved water.

4.3 Qualitative and Quantitative estimation of isolated DNA

The isolated DNA was qualitatively estimated by agarose gel electrophoresis. 0.7% agarose gel having ethidium bromide (EtBr) at the concentration 0.5µg /ml was made in 0.5X Tris Borate EDTA (pH=8) and casted in the electrophoretic apparatus along with chambered comb. The gel was allowed to solidify and the comb was carefully removed. Electrophoretic running buffer (0.5X TBE) was put into the tank so that the gel is fully immersed into the buffer.

The DNA samples were mixed with the 6 X loading dye. The samples were loaded into wells and allowed to run at 50 volts. The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

Quantitative Estimation of the Genomic DNA was done by the Spectrophotometric analysis of the sample. 1.5µl of DNA sample was diluted in 498.5µl Tris EDTA (300 times dilution). The absorbance of the sample was taken at 260nm, to determine the concentration of the sample. 1 OD is equivalent to 50µg/ml DNA sample so accordingly the concentration of the sample was calculated.

$$\text{Concentration (µg/ml)} = \text{O.D}_{260nm} \text{ X } 50\text{µg/ml X DilutionFactor}$$

The purity of the sample was checked by taking the ratio of its absorbance at 260nm and 280nm. The ratio more than 1.8 denotes RNA contamination, a ratio less than 1.8 denotes presence of proteins, phenol or other particles as contaminants while a ratio of 108 denotes pure DNA.

The DNA samples were then diluted to a concentration of 100 ng/µl by taking out appropriate amount of DNA sample in an eppendorf tube according to the quantity of DNA estimated in sample and then diluting it in Tris-EDTA buffer .

4.4 PCR –RFLP Analysis of *RASSFIA* gene for SNP rs2073498

PCR is a rapid process for *in vitro* amplification of desired DNA sequence by using specific primer so as to produce a large amount of desired DNA fragment of defined sequence length. RFLP is a technique that exploits variations in homologous DNA sequences. It refers to difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and proceeds to separate them by gel electrophoresis. Together these two techniques form the basis of PCR-RFLP analysis. This analysis was performed to determine the genotype of the G/T polymorphism of the *RASSFIA* gene. The 217 bp fragment encompassing the G to T polymorphic site in *RASSFIA* region was amplified by using specific primers 5′-GTA CAT CAG GGA CAG GGG GC-3′ (forward) and 5′-CAT GAA GAG GTT GCT GTT GAT C-3′ (reverse) (Kanzaki *et al*., 2005; Bayram, 2012).The reaction mixture was

placed in 200µl PCR tubes and the amplification was carried out in Thermocycler (T 100, Bio-Rad Laboratories, Inc.).

TABLE 5: PCR Reaction Setup Components

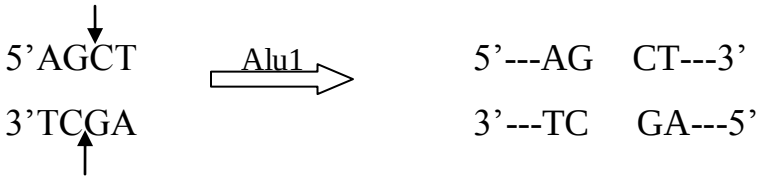
REAGENT	**STOCK CONCENTRATION**	**FINAL REACTION CONCENTRATION**	**QUANTITY USED**
Additive 1 BSA	1000µg/ml	100µg/ml	2.5µl
PCR Buffer (Mg Conc.)	10 X 15mM	1 X 1.5mM total	1.5µl
Primer (Forward)	10µM	0.5µM	1.25µl
Primer (Reverse)	10µM	0.5 µM	1.25µl
Taq Polymerase	3.0U/µl	0.8U	0.3µl
dNTP	10mM each	0.2mM each	0.5µl
PCR Grade Water			16.7µl
DNA Template	100ng/µl	400ng	1µl
TOTAL REACTION VOLUME = 25 µl			

TABLE 6: **Temperature Profile for PCR**

STEP	**TEMPERATURE**	**TIME**
STEP I : Initial Denaturation	95°C	5 min
STEP II : Denaturation	94°C	30 sec
STEP III : Annealing	63°C	30 sec
STEP IV: Extension	72°C	45 sec
STEP V	Step II to Step IV repeated 29 times	
STEP VI : Final extension	72°C	5min
STEP VII: Store	12°C	∞

The qualitative estimation of amplified DNA was done by agarose gel electrophoresis. 1.7% agarose gel having ethidium bromide (EtBr) at the concentration 0.5µg/ml and made in 0.5X Tris Borate EDTA (pH=8) was used for the purpose and the gel was run in 0.5X TBE as running buffer at 50V for 1.5 hour. 100bp ladder (G- Biosciences) was loaded in one of the wells for reference to band size of amplified DNA .Gel imaging was performed in UV light.

After confirmation of successful amplification, the PCR product was then digested overnight with *Alu1* enzyme at 37º C. *Alu1* is a restriction enzyme isolated from *Arthrobacterluteus*. Its restriction site is showed in following figure.



The reaction mixture consisted of 7.5µl water, 2.2µl 10X NEB4 buffer, 10µl PCR product and 0.3µl of 10U/µl *Alu1* enzyme with total reaction volume of 20 µl. The digested products were then electrophoresed on 2.5% agarose gel in 0.5X TBE, containing 0.5µg/ml EtBr and visualized under UV light. 50bp ladder (G- Biosciences) was used for reference to band size. Also, gel imaging was performed. PCR products with Ala at the polymorphic site were digested into two fragments, 158bp and 59bp; while those with Ser at the site were not digested because of absence of *Alu1* restriction site. Samples yielding 158bp and 5bp fragments were scored as Ala/Ala (wild type), those with single 217bp fragment as Ser/Ser (mutant), and those with 217bp, 158bp and 59bp fragments as Ala/Ser (heterozygous) (Kanzaki *et al.,* 2006; Bayram, 2012).

4.5 Statistical analysis

Data analysis was performed using SNP Analyzer 2 and Medical statistical software Medcalc. The differences of genotype and allele frequency between the cases and controls were determined using Pearson's-Chi-square (χ^2) test. To evaluate the deviation from Hardy-Weinberg equilibrium, the discrepancies between observed and expected genotype frequencies in patients and controls were compared using a χ^2 test with 2 degrees of freedom. If p is defined as

the frequency of the dominant allele and q is defined as the frequency of the recessive allele for a trait controlled by a pair of alleles, then Hardy-Weinberg equilibrium states that $p^2 + 2pq + q^2 = 1$. Crude Odds Ratio (OR) at 95% confidence interval was also calculated. All those *p* values which were less than 0.05 were taken to be significant. The differences in the distribution between cases and controls were tested using the $\chi 2$, and Fisher exact where appropriate. The crude odd ratios (ORs) were calculated by Wolf's method, The Odds ratios (ORs) with 95% confidence interval (CI) calculated were computed to estimate the association between certain genotypes or tobacco smoking and disease. Smokers were considered current smokers if they smoked up to one year before the date of diagnosis for cancer or up to the date of interview for controls. Information was collected on the number of cigarettes smoked per-day, the age at which the subject started smoking and the age at which the subject stopped smoking if the person was an ex-smoker (Kanzaki *et al*., 2006; Xiao *et al.* 2009).

RESULTS & DISCUSSIONS

5.1 Genotyping

DNA was isolated from blood samples (as discussed in section 4.2) using a simple and efficient procedure and the samples were run on 0.7% gel. This total isolated DNA was used as template in PCR.

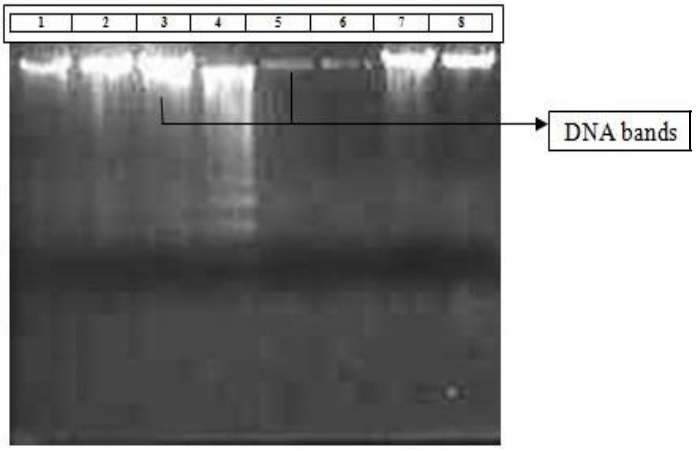


Fig. 9: Agarose gel electrophoresis picture for genomic DNA isolated from peripheral blood

After amplification, DNA fragments of 217bp (*RASSFIA*) were obtained as compared to 100bp ladder. The DNA bands were clearly and distinctly visible which indicated the successful amplification of *RASSFIA* by the primer combination used for the purpose (Fig. 10).

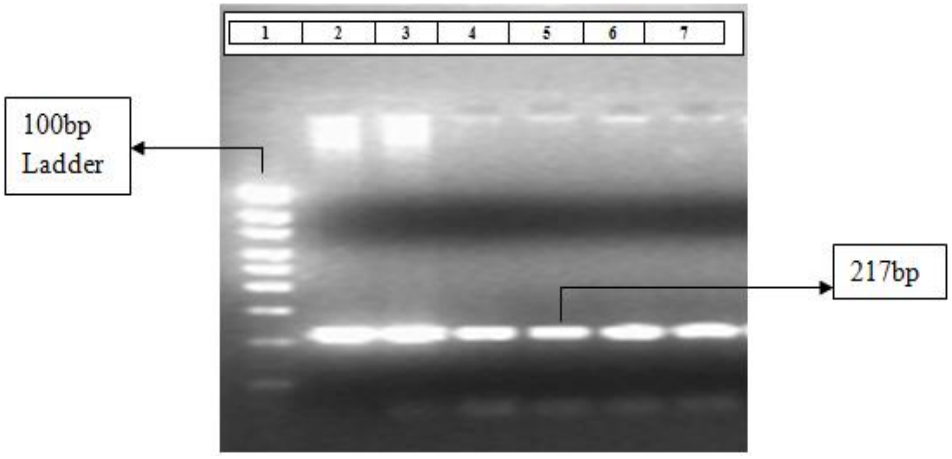


Fig. 10: Agarose gel electrophoresis picture for PCR amplified product for *RASSFIA*.

Lane1 – 100bp Ladder; Lane2-Lane7 – DNA products amplified for *RASSFIA* (217bp)

The PCR amplified products after being subjected to restriction digestion were separated on 2.5% agarose gel. The genotypic analysis of samples was done on the basis of fragments seen in the gel picture: 2 bands (158bp and 59bp) – Ala/Ala (Wild genotype); 3 bands (217bp, 158bp and 59bp) – Ala/Ser (Heterozygous genotype); 1 band (217bp) – Ser/Ser (Mutant genotype).

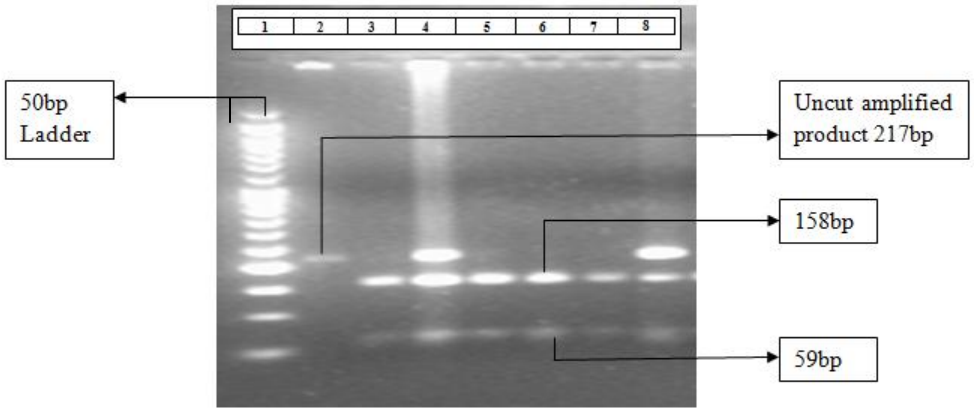


Fig.11: Agarose gel electrophoresis picture for restriction enzyme (*Alu1*) digested products, showing wild Ala/Ala and heterozygous Ala/Ser genotype. Lane 1- 50bp ladder**;** Lane 2- Uncut amplified product (217bp fragment); Lane 3, Lane 5-7 – Ala/Ala (wild) genotype ; Lane 4, Lane 8 – Ala/Ser (heterozygous) genotype.

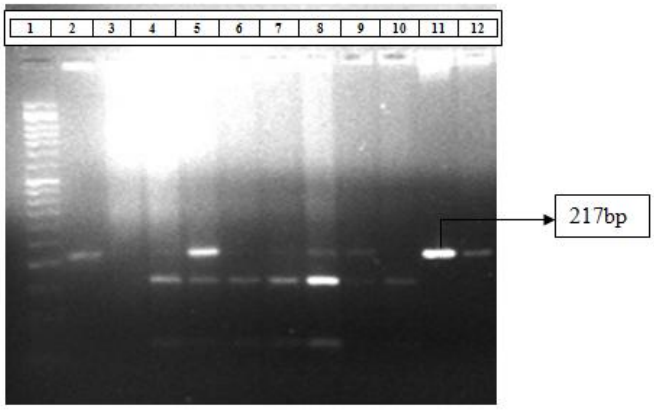


Fig 12: Agarose gel electrophoresis picture for restriction enzyme (*Alu1*) digested products, showing mutant Ser/Ser genotype. Lane 1- 50bp ladder; Lane 2- Uncut amplified product (217bp fragment); Lane 11-12 – Ser/Ser (mutant) genotype.

5.2 Epidemiology

The relevant characteristics of the subjects studied are shown in Table 7. The mean age was 56.45(±12.28) and 51.5(±10.35) among cases and controls. The ratio of number of people above and equal to age of 50 to the number of people below the age of 50 is much higher in cancer patients than controls. It may be perceived that lung cancer is more prevalent in age group of 50 years and above. The number of males in both cases and controls study were approximately same but only there was only 1 female in the control group (1.05%) as compared to 13 (14.44%) in cancer patients group. Considerably higher number of smokers were present in the patients group as compared to the controls group (78.89% and 44.21% respectively), which is in direct acceptance with the fact that tobacco smoking is one of the most prominent causes of lung cancer. The smokers were divided into two groups, heavy smokers were those with a smoking index (or Brinkman index) of more than 400 and light smokers were those with a smoking index of less than or equal to 400. 63.33% of the cases were light smokers and 26.66% were heavy smokers.

As many as 26.67% of cases had squamous cell carcinoma (SQCC), 14.44% had adenocarcinoma (ADCC), 16.66% had small cell lung carcinoma (SCLC) and 6.66% had other lung carcinomas (unknown histology). SQCC was found to be a little more prevalent than ADCC. This can be attributed to the fact that consumers of bidis, which are unfiltered and have high content of nicotine, are higher in number than consumers of cigarettes which are generally filtered and have low content of nicotine (Malson *et al.*, 2001). Smokers of low-yield cigarettes compensate for the low delivery of nicotine by inhaling the smoke more deeply and by smoking more intensely; such smokers may be taking up to 5 puffs/min with puff volumes up to 55 ml. Under these conditions, the peripheral lung is exposed to increased amounts of smoke carcinogens that are suspected to lead to lung adenocarcinoma (Wynder *et al.*, 1995); while the bidi smokers get their quota of nicotine by small puffs and they don't feel the urge to inhale the smoke in lungs, thus suffering from peripherally arising tumors such as SQCC. There is a considerable percentage of patients with SCLC also as most of the patients were smoking from twenty years or more (Table 7).

TABLE 7: Demographic characteristics in study subjects

VARIABLES	**CASES (n%)**	**CONTROLS (n%)**
	90	**95**
GENDER		
MALE	77(85.56%)	80(84.21%)
FEMALE	13(14.44%)	1(1.05%)
DATA N.A.	0(0.00%)	14(14.73%)
AGE		
<50	16(17.77%)	60(63.15%)
≥50	74(82.22%)	21(22.10%)
DATA N.A.	0	14
MEAN AGE (±SD)	56.45±12.28	51.5±10.35
RANGE (YEARS)	35-80	35-71
SMOKING STATUS		
NON SMOKERS	16(17.7%)	39(41.05%)
SMOKERS LIGHT SMOKERS HAEVY SMOKERS SMOKING INDEX UNKNOWN	71(78.89%) 57(63.33%) 24(26.66%) 9(10%)	 42(44.21%)
DATA N.A.	3(3.33%)	14(14.73%)
HISTOLOGY	**n(%)**	
SQCC	24(26.67%)	
ADCC	13(14.44%)	
SCLC	15(16.66%)	
OTHERS	6(6.66%)	
DATA N.A.	32(35.55%)	
STAGE	**n(%)**	
1	1(1.11%)	
2	4(4.44%)	
3	19(21.11%)	
4	21(23.33%)	
DATA N.A.	45(50%)	

TABLE 7 CONTINUE

TNM STAGING	**n(%)**	
T1	2(2.22%)	
T2	5(5.55%)	
T3	14(15.55%)	
T4	25(27.77%)	
DATA N.A.	44(48.88%)	

The stratification of lung cancer patients data according to the lung cancer stages shows maximum representation in 4th stage of lung cancer (23.33%), followed by stage 3 (21.11%), stage 2 (4.44%) and stage 1(1.11%), respectively. On stratification according to TNM staging, maximum number of cases were found in T4 stage (27.77%), T3 stage had 15.55% representation, T2 stage showed 5.55% representation while T1 stage had minimum representation of 2.22%. Rest cases couldn't be classified as data was unavailable for them (Table 7).

5.3 Prevalence of SNP Ala133Ser and its association with lung cancer in north- Indian population

The analysis of cases and controls for *RASSFIA* genotype is shown in Table 8. As the data depicts, the frequency of the Ser/Ser mutant allele was 4.44% and 2.10% respectively, thus there was almost twice representation of mutants in cases than controls, though the overall representation was very less. Lung cancer patients with both mutant alleles had 2 fold risk towards lung cancer (OR: 2.03, 95% CI; 0.35-11.55, *p* value = 0.41). However, due to low representation of mutants found in study, the data stands insignificant. For heterozygous/mutant population, OR is 0.90 (95% CI; 0.50-1.64), still not showing any significant association with lung cancer. Ala/Ala wild type allele covered most of the population, as shows in both the cases and control studies (63.33% and 61.05% respectively), whereas heterozygous Ala/Ser allele also

showed considerable representation of 32.22% and 36.84% in cancer cases and controls, respectively.

The allelic frequencies of case subjects (G 79.44%; T 20.55%) were almost same to those of the control subjects (G 79.44%; T 20.52%). The genotypic distribution of SNP in the cases was not in agreement with Hardy-Weinberg equilibrium (χ^2= 46.86, df= 2 , *p* value < 0.0001); For controls too, the genotypic frequencies were not in agreement with Hardy-Weinberg equilibrium (χ^2 =80.04, df=2, *p* value < 0.0001). The genotypic frequencies in the cases were not similar to that of the controls, differences being statistically significant (χ^2 =15.47, df=2, *p* value= 0.0004).

TABLE 8: Genotype & Allele frequencies of SNP A133S in study subjects

VARIABLES	**Controls (95)**	**Cases (90)**	**OR (95% CI)**	***p* value**
Ala/Ala (G/G)	58(61.05%)	57(63.33%)	1(reference)	
Ala/Ser (G/T)	35(36.84%)	29(32.22%)		
Ser/Ser (T/T)	2(2.10%)	4(4.44%)	2.03 (0.35-11.55)	0.41
Ala/Ser/Ser/Ser	37(38.94%)	33(36.66%)	0.90 (0.50-1.64)	0.74
ALLELE FREQUENCY				
G	151(79.47%)	143(79.44%)		
T	39(20.52%)	37(20.55%)		

∗ Degree of freedom = 2

In a study conducted on Japanese population, the proportion of Ala/Ala, Ala/Ser and Ser/Ser genotypes was 90.0, 9.1, 0.9% in controls and was 80.2, 18.8, 1.0% in the lung cancer patients, respectively. Lung cancer patients with the heterozygous Ala/Ser genotype and Ala/Ser+Ser/Ser genotype exhibited a significantly enhanced risk, with an OR of 2.59 (95% CI; 1.11–6.04) and 2.54 (95% CI; 1.04–6.22), respectively, with respect to the controls with the

Ala/Ala genotype (Kanzaki *et al*., 2006). Also, in a study conducted on Chinese population,the heterozygous mutation genotype was found to be a predisposing factor of lung cancer (OR: 2.34, 95% CI; 1.00–6.39). Also it was found that the heterozygous variant is a risk factor of lung adenocarcinoma (OR: 5.02, 95% CI; 2.10–14.54) (Xiao *et al*., 2009). Thus, there is a difference in the findings as there wasn't any such enhanced risk found with heterozygous variant genotype in this study.

5.4 Association between *RASSFIA* genotypes and different lung carcinomas

The analysis for *RASSFIA* genotypes in cancer patients for different lung carcinomas is shown in Table 9. The frequency of variant Ser/Ser mutant allele is highest in SQCC (3.33%), followed by others category, which includes carcinomas whose histology was unknown (1.11%) while the heterozygous Ala/Ser allele shows almost equal representation in ADCC, SCLC and SQCC (5.55%, 5.55%, 6.66%), with a much lower representation in other carcinomas (1.11%).

TABLE 9: Genotype frequencies of SNP A133S in different type of lung carcinomas

VARIABLES	ALA/ALA	ALA/SER	SER/SER
Cases	57(63.33%)	29(32.22%)	4(4.44%)
ADCC	8	5(5.55%)	0
SCLC	10	5(5.55%)	0
SQCC	15	6(6.66%)	3(3.33%)
OTHERS	4	1(1.11%)	1(1.11%)
UNKNOWN	20	12	0

"Others" includes those cases whose histology was unknown.

Table 10 presents the OR at 95% CI of SQCC cell type for *RASSFIA* genotypes. The association between Ser/Ser mutant genotype and SQCC lung cancer is highly significant with OR: 5.80 (95% CI; 0.88-37.90), showing an almost 6 times elevated risk of squamous cell carcinoma in individuals carrying both the mutant alleles. There is a highly significant 3 times

elevated relative risk of SQCC for mutant Ser/Ser genotype (OR: 2.9, 95% CI; 1.25-6.80, *p* value=0.01). The association for other lung carcinomas was not found to be significant.

TABLE 10: **Genotype frequencies of SNP A133S in SQCC patient subjects**

VARIABLES	**CONTROLS (95)**	**CASES (90)**	**SQCC (24)**	**OR (95%CI)***	***p* value**
ALA/ALA	58(61.05%)	57(63.33%)	15		
ALA/SER	35(36.84%)	29(32.22%)	6		
SER/SER	2(2.10%)	4(4.44%)	3	5.80 (0.88-37.90)	**0.04**
Ala/Ser/Ser/Ser			9	0.94 (0.37-2.36)	0.89

*OR of Controls= 1(Reference)

p value< 0.05

In a study conducted on Chinese population, the OR for Adenocarcinoma cell type was found to be 3.59 (95% CI; 1.35–9.59), posing a significantly 3 times higher risk factor. The heterozygous variant was found to be an insignificant risk factor, with OR of 1.714 (95% CI; 0.47–6.25) (Xiao *et al*., 2009).

5.5 Association between *RASSFIA* genotypes and smoking status

Cigarette smoke contains over 60 known carcinogens particularly polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene) and nitrosamines. Cytochrome P450 enzymes metabolically activate these polycyclic aromatic hydrocarbons and nitrosamines, which can then bind to DNA leading to DNA adducts. Glutothione-S-tranferases protect against DNA adduct formation by detoxifying carcinogen intermediates. DNA adducts are mostly repaired; however, chronic DNA adduct formation can lead to mutations in genes such as p53 that are central to lung cancer initiation (MacKinnon *et al.,* 2010).

In acceptance to the common facts, the cancer patients had higher percentage of smokers as compared to controls (78.88% and 44.21% in cases and controls respectively) (Table 11). Both cases and controls follow a pattern of highest representation of wild Ala/Ala genotype followed by heterozygous Ala/Ser genotype and least of mutant Ser/Ser genotype (11.11%, 6.66% and 0% for cases; 27.36%, 15.78%, 1.05% for controls, for Ala/Ala, Ala/Ser and Ser/Ser genotype respectively). Also, interestingly, there is mutant genotype representation only in the smokers section of cancer patients.

TABLE 11: Genotype frequencies of SNP A133S for smoking categories

VARIABLES		TOTAL	NON-SMOKERS	SMOKERS
CASES	**n**	**90**	**16(17.77%)**	**71(78.88%)**
	Ala/Ala	57	10(11.11%)	45(50%)
	Ala/Ser	29	6(6.66%)	22(24.44%)
	Ser/Ser	4	0(0.0%)	4(4.44%)
CONTROLS	**n**	**95**	**39(41.05%)**	**42(44.21%)**
	Ala/Ala	58	26(27.36%)	22(23.15%)
	Ala/Ser	35	15(15.78%)	16(16.84%)
	Ser/Ser	2	1(1.05%)	1(1.05%)

The OR of smokers suggests 2 fold increase in risk towards developing lung cancer, though *p* value was not significant [OR: 1.95 (95% CI; 0.20-18.55), *p* value 0.55]. The combined OR of heterozygous Ala/Ser and mutant Ser/Ser genotypes was found to be 0.97 (95% CI; 0.29-3.20) for non-smokers and 0.748 [95% CI; 0.33-1.65) for smokers, which shows there is no danger of the variants being weighed down by enhanced risk of lung cancer due to smoking because of the presence of mutant allele (Table 12).

TABLE 12: OR values of different genotypes of SNP A133S in study subjects for smoking categories

VARIABLES	OR (95% CI) NON-SMOKERS	*p* value NON-SMOKERS	OR (95% CI) SMOKERS	*p* value SMOKERS
Ala/Ala				
Ala/Ser				
Ser/Ser	0.84(0.03-22.34)	0.53	1.95 (0.20-18.55)	0.55
Ala/Ser/Ser/Ser	0.97(0.29-3.20)	0.96	0.74 (0.33-1.65)	0.73

In Japanese population study ,there was found to be a increased frequency of individuals with Ala/Ser genotype in smoking lung cancer patients (OR: 2.70, 95% CI; 1.06–6.83) and further an increased risk of the patients with heterozygous Ala/Ser genotype was observed, thus directly portraying smoking as a risk factor for lung cancer (Kanzaki *et al*., 2006). There have been studies conducted on north-Indian population previously which have proved smoking as the principle risk factor for causation of lung cancer in men (Gupta *et al*., 2001).

5.6 Association between *RASSFIA* genotypes and lung cancer stages

The patient population was scattered in all the different stages and sub stages of lung cancer, with highest number of patients being in stage 4 (23.33%) and stage 3 (21.11%). Here also the detailed genotypic analysis showed that wild type Ala/Ala genotype was represented mostly in each stage, followed by heterozygous Ala/Ser genotype and Ser/Ser genotype. Interestingly, 3 out of total 4 mutant Ser/Ser genotype cases found in cancer patients were at stage 4 of lung cancer (Table 13).

TABLE 13: Genotype frequencies of SNP A133S in lung cancer patient subjects for different stages of lung cancer

STAGE	n	ALA/ALA	ALA/SER	SER/SER
Cases	**90**	**57**	**29**	**4**
1	1(1.11%)	1(1.11%)	0	0
2	4(4.44%)	2(2.22%)	2(2.22%)	0
3	19(21.11%)	12(13.33%)	7(7.77%)	0
4	21(23.33%)	11(12.22%)	7(7.77%)	3(3.33%)

The OR of heterozygous Ala/Ser genotype for stage 3 was found to be 0.9 (95% CI; 0.3-2.6), while the absence of any Ser/Ser mutant genotypes ensured the same OR for combined Ala/Ser/Ser/Ser variant genotypes. For stage 4, Ser/Ser mutant genotype gives an OR of 7.9 (95% CI; 1.1-52.9) with a significant *p* value of 0.03 (Table 14).

TABLE 14: OR values of different genotypes of SNP A133S in cancer patient subjects for stage 3 and stage 4 of lung cancer

VARIABLES		OR(95% CI)	*p* value
STAGE 3	**Ala/Ala**	1 (Reference)	
	Ala/Ser	0.9 (0.3-2.6)	0.9
STAGE 4	**Ala/Ala**	1 (Reference)	
	Ser/Ser	7.9 (1.1-52.9)	**0.03**

p value <0.05

CONCLUSION

CONCLUSION

The present case/control study pertains to patients visiting the post graduate institute of Medical Education and Research, which is a referral center for patients from states like Haryana, Himachal Pradesh, Punjab, Uttar Pradesh, Jammu & Kashmir and Chandigarh. Following points were evident from our study:

- Our results show an association between the SNP rs2073498 (Ala133Ser) of *RASSFIA* gene and elevated risk of lung cancer, in North Indian population, though the data was not statistically significant due to small size of population studied.
- Statistically significant 6 times enhanced risk for Squamous cell carcinoma was found in individuals carrying both the mutant alleles.
- On stratification according to stages of lung cancer, a statistically significant OR suggested association of Ser/Ser mutant genotype with stage 4 of lung cancer.
- Results showed convincing evidence that *RASSFIA* polymorphism is an important modifying factor in determining susceptibility to lung cancer.
- Because our results suggest for the first time that the Ser133 allele of *RASSF1*A Ala133Ser polymorphism may be a genetic susceptibility factor for lung cancer in the North-Indian population, further independent studies with more number of cases and controls are required to validate the current findings.

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APPENDIX 1

1. **0.5M EDTA**: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionsed water, and then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.
2. **10% SDS**: Dissolved 1g of SDS in 10ml of deionsed water.
3. **100mM Tris-Cl (pH 8.0**): Dissolved 0.32g of Tris-Cl in 10ml of deionsed water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.
4. **10mg/ml Proteinase K**: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
5. **1mg/ml BSA**: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4 ^{o}C overnight.
6. **5% DMSO**: Mixed 50ml of 100% DMSO in 50ml of deionised sterile water. Sterilize the solution by autoclaving and stored at -20 ^{o}C.
7. **5M Sodium chloride (NaCl)**: Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilize the solution by autoclaving.
8. **5X TBE buffer**: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilized the solution by autoclaving.
9. **6X Loading dye**: Mixed 0.050g of bromophenol blue, 0.050g of xylene cyanol and 8g of sucrose. Mixed it in a small amount of TE buffer , then made up the volume upto 20ml by adding more TE buffer.
10. **Ethidium Bromide (10mg/ml)**: Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.
11. **Magnesium chloride ($MgCl_2$) (100mM)**: Dissolved 0.41g of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaving.
12. **Sucrose (1M)**: Dissolved 3.41g of sucrose in 10ml of deionised water and sterilized by autoclaving.
13. **TE buffer (pH 8.0)**: Added 1ml of 100mM Tris-Cl (pH 8.0) and 200µl of 0.5M EDTA solution to 8.8ml of deionised water. Sterilized the solution by autoclaving.
14. **Triton X- 100 (10%)**: 100µl of TritonX-100 mixed with 900µl of deionised water and mixed properly.